

Adjuvant for vaccine composition.

*Exhibit B1*

*B1*

The present invention relates to the field of vaccine compositions. More especially, the invention relates to new adjuvants used for increasing the immunogenicity of vaccine compositions.

There are a large number of antigens which, when injected into animals, will cause a production of antibodies which are specific to them. One of the principles of vaccination is to stimulate antibody production by the body of a man or an animal by administering chosen antigens thereto. The antibodies thus produced will then enable the body to defend itself against a subsequent infection. However, some antigens do not bring about sufficient stimulation of the immune system when they are administered alone. Hence an adjuvant which will enable the body's immune response to be increased has to be added to them in order to obtain a sufficient amount of antibody to be protective.

Among known adjuvants, aluminium hydroxide and aluminium phosphate, which are customarily used in human vaccines, may be mentioned. However, these compounds do not possess an adjuvant property with respect to all antigens. In particular, they do not enable the immunogenicity of influenza vaccine to be increased.

There is hence a need to be able to have adjuvants at one's disposal which enable the immunogenicity

of the antigens administered in a vaccine composition to be increased, without any risk of toxicity.

In addition, it is advantageous to have adjuvants at one's disposal which are capable of inducing an immune  
5 response that manifests itself in a production of secretory antibodies, such as IgAs.

To this end, the invention provides for the use of an amphipathic compound comprising a lipophilic group derived from a sterol linked to a cationic group, for the  
10 production of a vaccine composition.

A subject of the invention is also the use of such an amphipathic compound as adjuvant in the administration of a vaccine.

A subject of the invention is also a vaccine  
15 composition comprising at least one antigen, characterized in that it comprises, in addition, at least one amphipathic compound possessing a lipophilic group derived from a sterol linked to a cationic group.

A further subject of the invention is a product  
20 containing at least one antigen and one amphipathic compound comprising a lipophilic group derived from a sterol linked to a cationic group, as a combination product for use simultaneously, separately or staggered over time in vaccination.

25 Another subject of the invention is a method for inducing an immune response in a mammal, consisting in administering at least one antigen to the mammal,

characterized in that it consists in administering, in addition, at least one amphipathic compound comprising a lipophilic group derived from a sterol linked to a cationic group.

5 For the purposes of the present invention, the term amphipathic denotes a compound which possesses both a hydrophobic portion and a hydrophilic portion.

Among sterol derivatives capable of yielding the compounds according to the invention, cholesterol,  
10 phytosterol and ergosterol may be mentioned. Cholesterol derivatives are especially suitable.

The cationic group of the amphipathic compounds according to the invention can consist of a quaternary ammonium or an amine which can be protonated.

15 The lipophilic group is attached to the cationic group by means of an ester, ether, amide or carbamoyl link, among which ester, amide and carbamoyl links have the advantage that they can be hydrolysed in the cell.

The linkage between the 2 groups is preferably  
20 effected via a spacer arm consisting of a branched or unbranched alkyl chain comprising from 1 to 20 carbon atoms.

Among the compounds suitable for the purposes of the invention, there may be mentioned those mentioned in  
25 Patent US 5,283,185 and, in particular:

- cholesteryl-3 $\beta$ -carboxamidoethylenetrimethyl-  
ammonium iodide,

- cholesteryl-3 $\beta$ -carboxamidoethylenamine,

- cholesteryl-3 $\beta$ -oxysuccinamidoethylene-  
trimethylammonium iodide,

- 3 $\beta$ -[N-(N',N'-dimethylaminoethane)carbamoyl]-  
cholesterol,

5       - 3 $\beta$ -[N-(polyethylenimine)carbamoyl]cholesterol  
among which 3 $\beta$ -[N-(N',N'-dimethylaminoethane)carbamoyl]-  
cholesterol is especially advantageous.

The amphipathic compounds according to the  
invention may be obtained by condensation between a  
10 sterol derivative and a compound containing a cationic  
group, according to one of the methods described in  
"Advanced Organic Chemistry" Part B : Reactions and  
Synthesis (F.A. Carey and R.J. Sundberg - Plenum  
Publishing Corp.). More especially, some of the  
15 compounds according to the invention may be prepared  
according to the methods described in Patent US  
5,283,185.

The amphipathic compounds obtained in alcoholic  
solution can then be dispersed in water or in an aqueous  
20 buffer, and can yield a suspension of micelles or of  
liposomes. Advantageously, the amphipathic compounds of  
the invention are combined with a neutral lipid such as  
a phospholipid, for example dioleoylphosphatidylethanol-  
amine (DOPE) or dioleoylphosphatidylcholine (DOPC). This  
25 combination causes the amphipathic compounds according  
to the invention to organize themselves in the form of  
liposomes rather than micelles during the phase of  
dispersion in an aqueous environment. The molar

proportion of neutral lipid combined with the amphipathic compounds is preferably greater than 20%.

The products obtained according to the invention did not give rise to any acute toxicity reaction when  
5 they were inoculated into mice.

The antigen used to induce a protective immune response consists of any antigen customarily used in a vaccine composition, either alone or in combination with another antigen.

10 In particular, the amphipathic compounds according to the invention prove to be good immunoadjuvants when they are combined with the influenza virus vaccine comprising, in particular: the HA protein which is a haemagglutinin located at the  
15 surface of the influenza virus envelope, the NP protein which is a capsid nucleoprotein linked to the viral RNA and an M protein or protein "matrix" of the envelope.

Combination between the antigen whose immunogenicity it is desired to increase and the  
20 micellar or liposomal suspension of amphipathic compounds takes place spontaneously by hydrophobic and electrostatic interaction on mixing the constituents.

The vaccine compositions obtained possess good stability. However, the liposomal suspension appears  
25 preferable to the micellar suspension.

In addition, the liposomal suspension can be sterilized by filtration and lyophilized.

It is obvious that it is possible to add ingredients traditionally used in vaccines, such as water,

physiological saline or a buffer substance, to the vaccine compositions obtained.

Administration of the vaccine compositions obtained according to the invention may be performed by all the routes customarily used for the administration of vaccines, and in particular by the subcutaneous or intranasal route. It is also possible to choose a different route for the primary immunization and the booster immunization.

10 It is possible to administer separately the composition comprising the antigen and the composition containing the amphipathic compounds according to the invention; however, the administration of a liposomal composition of amphipathic compounds according to the invention combined with the antigen makes it possible not only to increase the humoral type immune response, but also to induce specific cytotoxic T lymphocytes.

A better understanding of the invention will be gained on reading the non-limiting examples which follow, reference being made to the figures.

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Brief Description of the Drawings

Figure 1 illustrates the reaction scheme for the production of DC chol. Figures 2 to 6 depict the results of tests of induction of cytotoxic T lymphocytes for each group of mice mentioned in Example 8.

25 Figure 7 depicts the results mentioned in Example 11.

Figures 8 and 9 depict the results mentioned in Example 12.

Figures 10 and 11 depict the results mentioned in Example 13.

Figures 12 and 13 depict the results mentioned in Example 14.

5        Figures 14 and 15 depict the results mentioned in Example 15.

Figure 16 depicts the results mentioned in Example 16.

2        Figure 17 depicts the results mentioned in Example 9.  
Σ        Figure 18 depicts the results mentioned in Example 1.

10      Example 1 : Synthesis of 3β-[N-(N',N'-dimethylaminoethane)-  
carbamoyl]cholesterol (DC chol)

DC chol is synthesized by reacting cholesteryl chloroformate and N,N-dimethylethylenediamine according to the scheme in Figure 1, as described in the paper by X. Gao and L. Huang (BBRC 179 (1) : 280-285).

A solution of cholesteryl chloroformate (2.25 g, 5 mmol in 5 ml of dry chloroform) is added dropwise to an excess of a solution of N,N-dimethylethylenediamine (2 ml, 18.2 mmol, in 3 ml of dry chloroform) at 0°C. After extraction of the solvent by evaporation, the residue is purified by 2 successive recrystallizations in absolute ethanol at 4°C, and dried under vacuum. 0.545 g of DC chol is thereby obtained in the form of a white powder. The structure of the compound was verified by NMR and mass spectrometry. The results obtained are in agreement with the data published in the paper cited above.

Example 2 : Preparation of a vaccine composition against influenza virus from a micellar suspension of DC chol  
2.3 mg

5           30 mg of DC chol obtained according to Example 1  
are dissolved in 100  $\mu$ l of ethanol. 75  $\mu$ l of the  
solution thereby obtained are injected via a Hamilton  
syringe into 3 ml of water kept stirring at 45°C. After  
a further 5 minutes of stirring at 45°C, the micellar  
10 suspension obtained is mixed with 200  $\mu$ l of monovalent  
vaccine against influenza virus (Strain A/Singapore)  
comprising, in particular, as antigens: the  
haemagglutinin HA, nucleoprotein NP and M protein.

The mixture obtained is divided into vaccine  
15 doses of 0.3 ml. Each dose comprises 5  $\mu$ g of HA and  
2.3 mg of DC chol.

Example 3 : Preparation of a vaccine composition against influenza virus from a micellar suspension of DC chol  
20 0.45 mg

The procedure is as in Example 2, starting from  
6 mg of DC chol obtained according to Example 1.

25 Example 4 : Preparation of a suspension of liposomes  
consisting of DC chol combined with dioleoyl-  
phosphatidylethanolamine (DOPE)

18 mg of dioleoylphosphatidylethanolamine (DOPE) and 4.5 mg of DC chol obtained according to Example 1, which is dissolved in 3 ml of chloroform, are mixed.

The chloroform is evaporated off under vacuum to form a lipid film, which is subjected to dessication under vacuum and then resuspended in 3 ml of water.

After hydration for 24 hours at 4°C, the dispersion is subjected to sonication for 5 to 10 minutes in an ultrasound bath (Laboratory Supplies - Hicksville - N.Y.) to form liposomes.

This suspension is stable for at least 6 months at 4°C.

Example 5 : Preparation of a suspension of liposomes consisting of DC chol combined with dioleoylphosphatidylcholine (DOPC)

The procedure is as in Example 4, replacing the 18 mg of DOPE by 18 mg of dioleoylphosphatidylcholine (DOPC).

A liposomal suspension which is stable for at least 6 months at 4°C is obtained.

Example 6 : Preparation of a vaccine composition against influenza virus from a DC chol/DOPE liposomal suspension

3 ml of a liposomal suspension obtained according to Example 4 are mixed with 0.2 ml of strain

A/Singapore monovalent influenza vaccine containing the equivalent of 50 µg of the antigen consisting of the haemagglutinin HA.

The mixture obtained is then divided into 10 vaccine doses of 0.3 ml, each containing 5 µg of HA and 0.45 mg of DC chol.

Example 7 : Preparation of a vaccine composition against influenza virus from a DC chol/DOPC liposomal suspension

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3 ml of a liposomal suspension obtained according to Example 5 are mixed with 0.2 ml of strain A/Singapore monovalent influenza vaccine.

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The mixture obtained is then divided into 10 vaccine doses of 0.3 ml, each containing 5 µg of HA and 0.45 mg of DC chol.

Example 8 : Immunization

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5 groups of 4 Balb/c mice are immunized by 3 subcutaneous injections performed on D0, D21 and D36 with the following vaccine compositions:

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Group A: 0.3 ml of diluted strain A/Singapore monovalent influenza vaccine containing 5 µg of HA in 0.3 ml of PBS,

Group B: 0.3 ml of vaccine composition  
obtained according to Example 2,

Group C: 0.3 ml of vaccine composition  
obtained according to Example 3,

5 Group D: 0.3 ml of vaccine composition  
obtained according to Example 6,

Group E: 0.3 ml of vaccine composition  
obtained according to Example 7.

10 Example 9 : Assay for anti-HA antibodies

In order to perform the assays for neutralizing  
antibodies, the sera of mice are sampled on D21, D36 and  
D51, and the titration of anti-HA antibodies is  
15 performed by means of the technique of inhibition of  
influenza virus-induced haemagglutination.

Table 1 below illustrates the results obtained  
for each group of mice after one injection, and Table 2  
the results after 2 injections.

Table 1

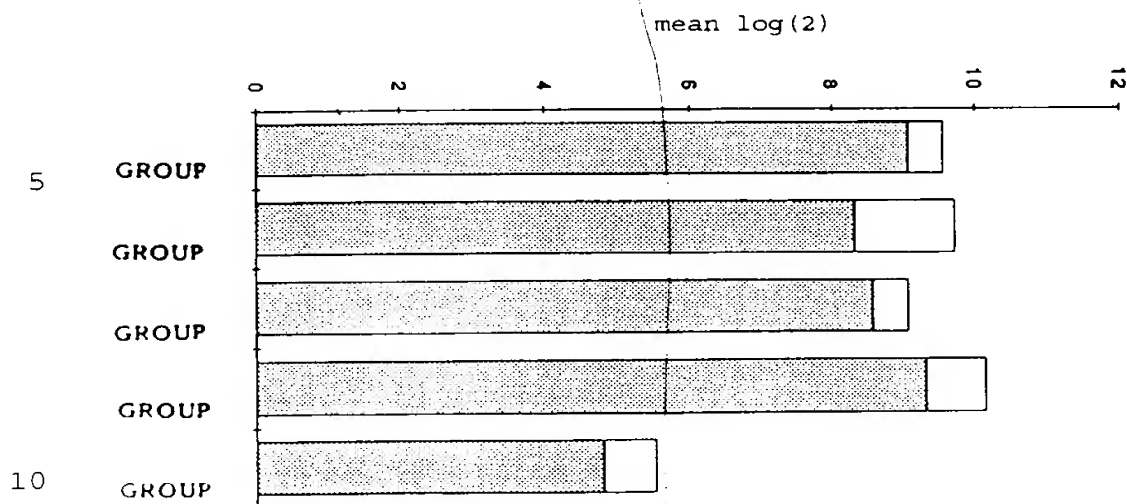
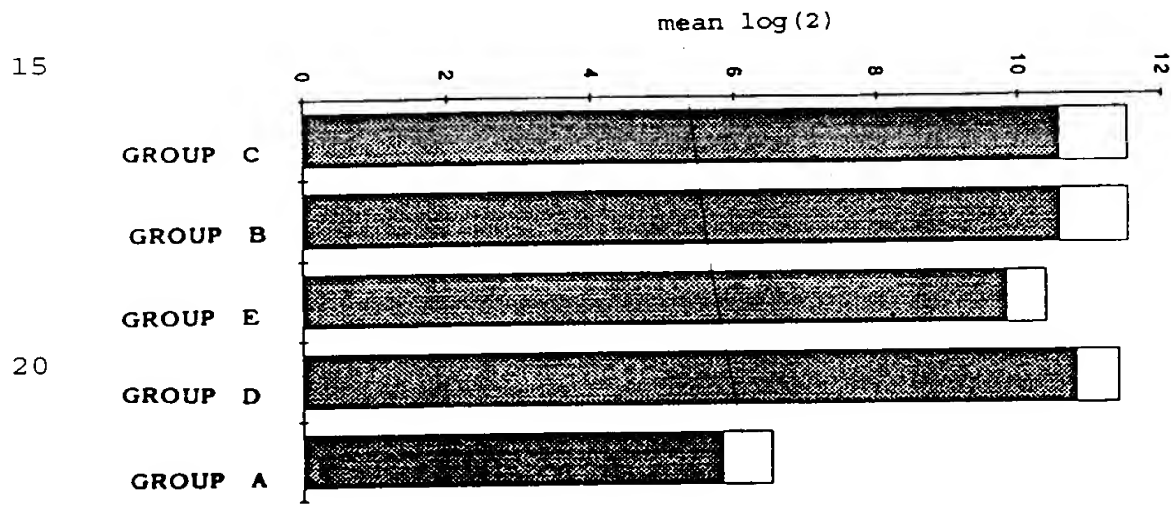


Table 2



25 The titres of neutralizing antibodies in the mouse sera are presented in the form of log<sub>2</sub> of the highest dilution inducing haemagglutination inhibition.

These results show clearly the adjuvant role played by DC chol. In effect, the level of anti-HA antibodies is markedly higher for the groups of mice which have received DC chol in comparison with mice 5 which have received the vaccine without adjuvant (Group A).

It is important to note that the levels of neutralizing antibodies in Groups B, C, D and E are greater than the level of neutralizing antibodies in 10 Group A, even after a single injection of the different vaccine compositions. These titres increase slightly more and stabilize after the second injection. The results obtained after the 3rd injection are substantially equal to those for the 2nd injection (not 15 shown).

Tests carried out with a trivalent influenza vaccine comprising strain A/Texas, strain B/Panama and strain A/Beijing also demonstrated the adjuvant power of DC chol.

20

Example 10 : Demonstration of the induction of cytotoxic T cells

The spleen cells of the mice of each of the 25 groups mentioned in Example 8 are removed on D51.

These effector cells are restimulated in vitro in the presence of syngeneic stimulatory cells infected with the strain A/Singapore virus corresponding to the

vaccine tested. The specific cytotoxic function of these stimulated cells is demonstrated using as target cells the P815 mastocytoma line sensitized by incubation with a peptide which is a CTL epitope of the haemagglutinin of the virus (specific response against the HA) or with a peptide which is a CTL epitope of the nucleoprotein of the virus (specific response against the NP). Non-specific lysis (background) is measured on P815 cells which are unsensitized or sensitized with a peptide which is a CTL epitope of the HIV virus (V3MN).

Lysis of the target cells is measured by a radioactive technique based on loading of the target cells with Cr-51 and on release of this radioelement during cell lysis.

The results presented in Figures 2 to 6, which illustrate the percentage cytotoxicity in terms of the ratio of effector cells to target cells for each of the groups of mice tested, show that it is especially advantageous to use a liposomal composition of DC chol especially in combination with a neutral lipid, since this composition makes it possible to induce specific cytotoxic T lymphocytes in addition to the humoral type immune response obtained as a result of the adjuvant action of the DC chol.

Example 11 : Study of the immune response as a function of the dose of DC chol used

Vaccine compositions of 300  $\mu$ l are prepared,  
5 each containing strain A/Singapore monovalent influenza vaccine and containing either 15 or 5  $\mu$ g of HA, in combination with DC chol/DOPC liposomes at a variable concentration. The preparation of liposomal suspension is carried out in a manner similar to that of Example 4,  
10 replacing the 18 mg of dioleoylphosphatidylethanolamine (DOPE) by 13.5 mg of dioleoylphosphatidylcholine (DOPC) and taking up the lipid film obtained with an amount of water which varies according to the desired DC chol concentration.

15 The vaccine compositions obtained are injected into groups of 5 8- to 10-week-old female Balb/c mice on D0 and D28. The sera are sampled on D42 and the anti-HA antibodies are assayed by the agglutination inhibition (HAI) technique.

20 The following compositions are tested in this way:

- 15  $\mu$ g of HA + 0  $\mu$ g of DC chol
- 15  $\mu$ g of HA + 400  $\mu$ g of DC chol
- 5  $\mu$ g of HA + 0  $\mu$ g of DC chol
- 25 5  $\mu$ g of HA + 25  $\mu$ g of DC chol
- 5  $\mu$ g of HA + 50  $\mu$ g of DC chol
- 5  $\mu$ g of HA + 100  $\mu$ g of DC chol

5 µg of HA + 200 µg of DC chol

5 µg of HA + 300 µg of DC chol

5 µg of HA + 400 µg of DC chol

The results expressed in the form of log base 2  
5 of the highest dilution inducing haemagglutination  
inhibition are depicted in Figure 7, and show that only  
above 100 µg of DC chol/dose is the maximum benefit of  
the adjuvant effect of DC chol obtained.

10        Example 12 : Demonstration of the induction of  
different antibody isotypes

A comparative study is carried out of the  
antibodies induced in 3 groups of 5 8- to 10-week-old  
15 female BALB/c adult mice which have received 2  
subcutaneous injections performed on D0 and D28 with the  
following vaccine compositions:

20        1st group: 0.3 ml of diluted strain A/Singapore  
monovalent        influenza        vaccine  
containing 5 µg of HA in 0.3 ml of  
PBS,

25        2nd group: 0.3 ml of diluted strain A/Singapore  
monovalent        influenza        vaccine  
containing 5 µg of HA with 0.1 mg of  
aluminium hydroxide as adjuvant,

3rd group: 0.3 ml of vaccine composition  
obtained according to Example 11 and

containing 5  $\mu$ g of HA and 400  $\mu$ g of  
DC chol in combination with DOPC.

The sera of the mice are sampled on D28 and on  
5 D42 and the IgG1 and IgG2 produced are assayed by the  
ELISA technique.

The results obtained in the primary response are  
depicted in Figure 8 and those obtained in the secondary  
response are depicted in Figure 9.

10 These results illustrate the adjuvant effect of  
DC chol, both in the primary response and in the  
secondary response, in comparison to aluminium hydroxide  
which is an adjuvant of the prior art. It will be noted,  
in addition, that there is a large increase in the IgG2a  
15 produced, signifying that DC chol acts by promoting the  
development of TH1 type lymphocytes.

Example 13 : Demonstration of the action of DC  
chol in elderly mice

20

The same experiment is carried out exactly as in  
Example 12 but, in this instance, the mice tested are 20  
to 22-month-old mice which are more difficult to  
stimulate.

25 The results obtained in the primary response are  
depicted in Figure 10 and those for the secondary  
response in Figure 11.

Here too, DC chol has an adjuvant effect both for  
the primary response and for the secondary response,

and in particular with respect to the induction of IgG2a antibodies.

Example 14 : HAI levels in adult mice and in  
5 elderly mice

In the tests carried out in Examples 12 and 13, the neutralizing antibodies are determined in the immunized mice by the haemagglutination inhibition (HAI)  
10 test.

The results obtained in the primary response are illustrated in Figure 12 and those for the secondary response in Figure 13. The titres obtained, expressed in the form of log base 2 of the highest dilution  
15 inhibiting haemagglutination, shows clearly the adjuvant role of the DC chol/DOPC liposomes both in adult mice and in elderly mice.

Example 15 : Intranasal immunization  
20

Vaccine compositions are prepared as described in Example 11, in this instance of 50 µl comprising 5 µg of HA (in the form of strain A/Singapore monovalent influenza vaccine) combined with 200 µg of DC chol  
25 present in the form of DC chol/DOPC (in a mass ratio of 1 to 4) liposomes.

Two groups of 4 BALB/c mice are immunized intranasally twice with an interval of 4 weeks.

The 1st group of mice ( $G_1$ ) receives 50  $\mu$ l of the vaccine composition containing 200  $\mu$ g of DC chol, whereas the second group ( $G_2$ ) receives 50  $\mu$ l of the same monovalent influenza vaccine but without adjuvant. The  
5 immune responses of each group are analysed by ELISA assay of the sera sampled 3 weeks after the booster immunization.

The results, expressed in the form of log base 10 of the ELISA titre, are depicted in Figure 14 as  
10 regards the serum IgG and in Figure 15 as regards the serum IgA.

It will thus be noted that, in a protocol of strict mucosal administration, the DC chol/DOPC liposomes enable the local and general immune responses  
15 to be increased by a factor of at least 2.

The adjuvant role performed by the DC chol/DOPC liposomes in the general immune responses was also observed when, instead of measuring the IgG and IgA titres by ELISA, the anti-HA antibody titres were  
20 measured by HAI; the increase in the local immune response was also observed in an ELISPOT count of the number of cells secreting IgG and the number of cells secreting IgA in the lungs of the immunized mice, and also in the determination of the ratio of the level of  
25 specific IgG (or of IgA) to the level of total IgG (or of IgA, respectively) measured by ELISA in the nasopharyngeal lavage fluids of the immunized mice.

Example 16 : Immunization by combined  
subcutaneous and intranasal administration

Two groups of 4 BALB/c mice are immunized twice  
5 with an interval of 4 weeks with, on each occasion, the  
following vaccine compositions:

- Group 3 ( $G_3$ ): - a vaccine composition of  
300  $\mu$ l injected subcutaneously comprising 4  $\mu$ g of HA (in  
the form of strain A/Singapore monovalent influenza  
10 vaccine) combined with 6  $\mu$ g of DC chol (in the form of  
DC chol/DOPC liposomes), and

- a vaccine composition of 50  $\mu$ l  
administered intranasally comprising 0.25  $\mu$ g of HA (in  
the form of strain A/Singapore monovalent influenza  
15 vaccine) combined with 6  $\mu$ g of DC chol (in the form of  
DC chol/DOPC liposomes),

- Group 4 ( $G_4$ ): - a vaccine composition of  
300  $\mu$ l injected subcutaneously comprising 4  $\mu$ g of HA  
combined with 200  $\mu$ g of DC chol, and

20 - a vaccine composition of 50  $\mu$ l  
administered intranasally comprising 0.25  $\mu$ g of HA  
combined with 200  $\mu$ g of DC chol.

The sera of the immunized mice are sampled  
3 weeks after the booster administration and, for each  
25 mouse, the level of neutralizing antibodies is assayed  
by the HAI haemagglutination inhibition technique.

The results obtained, expressed in the form of log base 2 of the titre, are depicted in Figure 16, and show that, in a protocol of mixed administration combining the subcutaneous and intranasal routes, 5 DC chol has an adjuvant effect, and that the increase in the dose of DC chol from 6 to 200 µg enables the immune response to be increased.

An identical dose effect could be observed when, instead of measuring the level of anti-HA antibodies by 10 the HAI technique, the serum IgG was measured by the ELISA technique or the number of cells secreting IgG and the number of cells secreting IgA was measured in the lungs of the immunized mice (by the ELISPOT technique), or the ratio of the amounts of specific IgG (or IgA) to 15 the amounts of total IgG (or IgA, respectively) was measured in the nasopharyngeal lavage fluids of the immunized mice.

Results similar to those described in Examples 11 to 16 were obtained by replacing DOPC by 20 DOPE, as well as by varying the DC chol/neutral lipid ratio, maintaining the molar proportion of neutral lipid combined with DC chol at not less than 20%.